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**Enhanced Delivery of Naproxen to the Viable Epidermis from an Activated poly N-isopropylacrylamide (PNIPAM) Nanogel: Skin Penetration, Modulation of COX-2 Expression and Rat Paw Oedema**

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**Abstract:** Stimulus-responsive drug-loaded poly (N-isopropylacrylamide) nanogels were examined as a means of enhancing the delivery of naproxen into skin *ex vivo*. Following massaging into skin, the epidermis was probed (with and without base activation) for depth penetration and transdermal delivery of drug, and anti-inflammatory activity in the relative levels of COX-2 expression. Rat paw oedema testing was used to determine anti-inflammatory effects *in vivo*. When activated by sodium carbonate, particle size reduced by 19%. Tape stripping revealed significantly greater delivery of naproxen into the epidermis for the activated nanogel and the steady state flux was enhanced 2.8-fold. With base-activation COX-2 was 50% lower than non-activated, and this trend was confirmed by immunostaining, and by the reduction of rat paw swelling which provided *ex vivo* – *in vivo* corroboration. A mechanism of action is proposed. In conclusion, stimulus-responsive nanogels have potential for enhancing dermal drug delivery and therapeutic outcomes in inflammatory skin diseases.

**Keywords:** naproxen, PNIPAM, nanogel, COX-2, anti-inflammatory

## Background

Nanoparticles are widely evaluated in novel drug delivery systems.<sup>1-3</sup> Nanogels are sub-micron swollen nanoparticles composed of a hydrogel – a crosslinked hydrophilic network of synthetic polymers or biopolymers.<sup>4</sup> Nanogels undergo abrupt, reversible changes in volume in response to minor changes in the environment, such as temperature, pH, ionic strength, electric field, light intensity, as well as specific chemical stimuli.<sup>5,6</sup> Polymers based upon N-isopropylacrylamide (PNIPAM) have been widely used to prepare temperature-responsive hydrogels which possess a range of interesting physicochemical properties: low viscosity, very high surface area, pH and rapid thermal sensitivities. Such properties give them potential in ‘smart’, ‘controlled’ and ‘regulated’ drug delivery applications. For example, below the critical solution temperature (LCST) of approximately 32 °C, PNIPAM swells in water and imbibes any dissolved solute (drug); whereas above the temperature the structure collapses expelling water and solute, a process assisted by a decrease in internal hydrophilic environment.<sup>7</sup> Changes in pH cause a similar response.

Skin inflammation, erythema and pain is generally mediated by an upregulation in the local production of eicosanoids, in particular the prostaglandin PGE-2, which is biosynthesized by the action of cyclooxygenase-2 (COX-2) – the target for anti-inflammatory drugs, including non-steroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors.<sup>8</sup> Topical delivery, where appropriate, allows for localised and reduced doses, and also avoids the potential for gastrointestinal irritation. However, the skin has evolved to be an efficient barrier to the ingress of xenobiotics including NSAIDs and this causes difficulties for the development of efficacious transdermal and local, targeted dermatologically active drug delivery systems. Numerous strategies have been employed to overcome the

barrier including microneedles.<sup>9</sup> The use of nanogels for topical applications has been studied previously<sup>10,11</sup> for the delivery of estradiol, minoxidil and caffeine.<sup>2,12,13</sup> The size and release properties of nanogels are the essential key when designing nanogel delivery systems, and the sharp phase transition or lower critical solution temperature of PNIPAM nanogels in aqueous solution, approximately 32 °C, is highly appropriate as the average skin surface temperature is 32-34 °C and has been shown to provide stimulus-responsive enhanced topical drug delivery of and caffeine and methotrexate.<sup>13,14</sup> Topical naproxen gels have been reported with limited success.<sup>15</sup> Lipid nanoparticles have recently been previously reported for the delivery of naproxen into skin, although no evidence was provided to demonstrate anti-inflammatory effect.<sup>16</sup>

The aim of the present study was to evaluate environmental modulation on the ability of responsive PNIPAM nanogels to deliver naproxen into and across the skin and assess consequent biological effects in the keratinocytes of the viable epidermis, *ex vivo* in the modulation of epidermal COX-2 *ex vivo*<sup>17,18</sup> and verification of the effect *in vivo* using the rat paw oedema model.<sup>19</sup>

## **Methods**

### **Materials**

Naproxen was obtained from Abdi Ibrahim Pharm (Istanbul, Turkey). N-isopropylacrylamide 97% (NIPAM), butyl acrylate 99% (BA), N,N-methylenebisacrylamide 99% (BIS), potassium persulphate 99.9%, citric acid (CA), sodium carbonate (SC), Radioimmunoprecipitation assay buffer (RIPA buffer, comprised of 50 mM tris-HCl (pH 7.4), 1 mM EDTA, 1% Triton X-100, 1% sodium

deoxycholate, 150 mM NaCl, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride serine protease inhibitor), Hanks balanced salt solution (HBSS), gentamycin sulphate, Ponceau S, PBS + 0.05% TWEEN, aprotinin, leupeptin, anti-actin antibody, sodium azide, SDS 10%, paraffin wax pellets, phosphate buffer saline pH 7.4 (PBS), methyl green, distyrene/plasticizer/xylene (DPX mountant) and  $\lambda$ -carrageenan were all purchased from Sigma-Aldrich, Poole, UK. Trifluoroacetic acid, triethylamine and HPLC-grade solvents were purchased from Fisher Scientific (Loughborough, UK). Primary COX-2 antibody (#4842) was purchased from Cell Signalling Technology (Boston, USA). Horseradish peroxidase (HRP)-labelled anti-rabbit polymer, DAB chromagen plus substrate was from Dako UK (Ely, England). Western blocking reagent was from Roche Diagnostics, GmbH (Mannheim, Germany). Rainbow Marker (10-250 Kd), anti-mouse HRP, (HRP)-linked antibody were from Amersham Biosciences Ltd (Buckinghamshire, UK). Dura substrate was from Perbio, Cramlington, UK, and 3,3'-Diaminobenzidine (DAB) chromagen-AB substrate was from Abcam, Cambridge, UK. Freshly excised porcine ears were obtained from a local abattoir prior to steam cleaning, immediately following slaughter.

### **Synthesis of Naproxen-loaded PNIPAM Nanogels**

PNIPAM nanogels were prepared by surfactant-free emulsion polymerisation in deionised water at 60 °C, under nitrogen. For the blank nanogel (BN), potassium persulphate was dissolved in 200 mL deionised water in a 500 mL, three-necked round-bottomed flask and stirred continuously at 120 rpm. NIPAM (2g) and BA (0.25 mL) as co-monomers, and *N,N*-methylenebisacrylamide (7.5%) as the crosslinker were dissolved in 200 mL deionised water and mixed with the potassium persulfate

under stirring and then added to the reaction vessel. After 6 h the crosslinked PNIPAM nanogels were cooled to room temperature and purified by multiple washing/centrifugation /washing at 50,000 x g, 20°C for 1 h in an Avanti<sup>®</sup> J-25 centrifuge (Beckman, Fullerton, CA), with distilled water, until the conductivity was less than 1  $\mu\text{S cm}^{-1}$ .<sup>20</sup> For NN, 500mg naproxen was dissolved in the potassium persulfate solution beforehand with a pH of 6, whereas for NNCA the potassium persulfate solution containing naproxen was also adjusted to pH 2.5 with citric acid prior to mixing base (Table 1).

Particle diameter and size distribution of the purified nanogel particles were determined by a laser diffraction system, Malvern Mastersizer 2000 (Malvern Instruments Ltd., Malvern, UK). Drug-loaded nanogel suspension was ultra-centrifuged at  $150000 \times g$  (Beckman Coulter J25), for 2 hours to separate the free drug from encapsulated drug. The clear supernatant above the nanogels was removed by decanting before being assayed for dissolved naproxen by HPLC. The reproducible product - a viscous precipitate - was used for further experimentation. Confocal images were determined by scanning laser microscopy (CSLM, MRC 1024 UV, Bio-Rad, Hemel Hempstead, UK) using a 60  $\times$  water immersion lens (Nikon, Badhoevedorp, Netherlands).

### **Percutaneous penetration**

Freshly excised porcine ear skin was liberated from the underlying cartilage by blunt dissection, whilst being continually bathed in Hanks buffer and 2 x 2 cm<sup>2</sup> membranes were mounted on the pre-greased flanges of all-glass Franz-type diffusion cells<sup>9</sup>, with nominal diffusional diameter of 1 cm and receptor phase volume of 3 mL, which was

degassed Hanks buffered salt solution (HBSS)-gentamycin. Receptor compartments were filled with degassed Hanks buffer, and micro stirrers added. The complete assemblies were placed on a submersible stirrer plate in a water bath set at 37°C. After equilibration for 15 min the skin was dosed using the flattened end of a glass rod to gently massage 200mg of the viscous nanogel slurry onto the skin using 10 rotations. An equimolar naproxen solution comparator was prepared at pH6. In the case of the 'activated' samples, an aliquot of either 100 µL of 0.1M sodium carbonate (or triethylamine for Western blotting) was added and massaged by a further 10 rotations (NNCA+SC and NNCA+TEA). When required, the cells were dismantled and the skin membranes recovered.

### **Tape stripping**

Tape stripping is a commonly used technique for the comparative assessment of xenobiotic penetration into skin.<sup>9</sup> Regular Sellotape strips, approximately 25mm in length, were pressed onto the pig ear skin and then removed using forceps, before being placed into clean glass vials containing 2 mL methanol. This was repeated 10 times before methanol (2 mL) and the vials left for 24 h on a shaker (Infors HT Labotron, Germany) at room temperature. Tapes were removed and the solutions added to HPLC vials. The remaining skin of the diffused area was excised, cut into small pieces and also extracted with methanol. The drug amounts in the first strips were not included in the analysis to eliminate any possible surface contamination.



## **HPLC Analysis**

Naproxen was determined using an Agilent 1100 automated HPLC system with a Phenomenex Kingsorb C18 column (150 x 4.6 mm, 5  $\mu$ m). The mobile phase was acetonitrile/water (adjusted to pH 2.5 with trifluoroacetic acid) (60:40 v/v) run isocratically at a flow rate of 1.0 mL min<sup>-1</sup>. UV detection was at 271 nm and the retention time of naproxen was 7.8 min.

## **Western blotting analysis of COX-2**

Semi-quantitative modulation of COX-2 in skin as a consequence of dosing with nanogels was determined by Western blotting.<sup>21,22</sup> After 6 or 10h skin samples were recovered from the diffusion cells, gently cleansed with de-ionized water before being homogenized (Silverson, Chesham, UK) in as RIPA lysis buffer plus an additional 5  $\mu$ g mL<sup>-1</sup> of the protease inhibitors aprotinin and leupeptin. After 15 min incubation on ice, the lysates were centrifuged at 14000 x g for 2 x 15min and the supernatant stored at -80 °C until required.

The protein content of the lysates was determined using the Biorad D/C protein assay kit (Bio-Rad Laboratories, Hercules, CA). Aliquots of 30  $\mu$ g proteins with one channel for the Rainbow marker were separated by SDS-PAGE, transferred to nitrocelullose membranes using the Trans-Blot electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA), and briefly stained with Ponceau S to verify effective transfer. Immunoblots were incubated for 1 h in the blocking solution containing 5% (w/v) commercial Marvel milk powder (Premier Foods, St Albans, UK) at room temperature. After washing, the membrane was probed overnight at 4 °C with COX-2 antibody at 1:1000 in (1:20 and 1:100 western blocking reagent (Roche)

and sodium azide respectively made up to volume with TBS tween). Membranes were then incubated for 1 h with HRP-conjugated anti-rabbit. For  $\beta$ -actin, membranes were probed with mouse anti-actin antibody and anti-mouse (Neomarkers Ab-10) for 1 h each at room temperature. After 3x10 min washes in TBS Tween, they were exposed to freshly prepared Dura Substrate for chemiluminescence for 20 min before performing autoradiography.<sup>18</sup>

### **IHC analysis of COX-2 expression**

Visualisation of relative COX-2 levels *in situ* were determined based on a previously reported method<sup>17,18</sup> skin samples were cut into 5 x 2 mm sections after 3, 6, and 10 h ( $n = 3$ ) and placed in 4% formaldehyde then left to fix for 24 h. The skin was immersed sequentially in 70% to 90% to 100% ethanol solutions to displace all the water from the tissue, then into four chloroform baths to displace the alcohol. The skin sections were subjected to immersion in a series of three molten wax baths attached to a vacuum to eliminate the remaining chloroform, before being embedded in a paraffin wax block. The skin was then cut using a Shandon Finesse microtome and sections were transferred onto pre-cleaned regular micro-slides (Surgipath, Peterborough, UK). After re-hydration with ethanol and equilibration in PBS, specimens were blocked with PBS Tween for 15 min. The COX-2 primary antibody was then applied diluted 1:50 in PBS and stored overnight at 25 °C in a humidified chamber. The slides were then washed with PBS for 2 x 5 min and incubated in HRP labelled anti-rabbit for 2 h at 25 °C. Slides were washed in PBS for three min followed by 2 x 5 min washes in PBS/Tween. An aliquot (0.75  $\mu$ L) of visualization solution (DAB chromagen-AB substrate) was applied for 10 min. Following another washing in deionised H<sub>2</sub>O, the slides were counter-stained with 0.5% methyl green

for 3 min. Finally, sections were rinsed in distilled water and dried in an oven at 40 °C. DPX mountant was used to cover slips ready for microscope analysis.

### **In vivo rat paw oedema**

The carrageenan-induced inflammation in the rat paw model of oedema formation and hyperalgesia<sup>19</sup> was used to examine the *in vivo* effects of the nanogel formulations in male albino Wistar rats weighing 180–220 g. The experimental protocol was approved by the Local Ethics Committee for Animal Experiments, Ege University, Turkey, approved project number 2009-3. The animals were housed in standard environmental conditions and fed with standard rodent diet with water *ad libitum*, and all procedures were carried out in accordance with the institution's animal subject review committee. Inflammation was introduced in rats under anesthesia by using 100 µL of a 1% (w/v)  $\lambda$ -carrageenan solution in saline. The solution was injected into the plantar surface of the left hind paw of the rats and the contra-lateral paw received the same volume of sterile saline solution. To evaluate the topical anti-inflammatory activity of the formulations, three groups of animals (n = 6) with carrageenan-induced paw oedema were examined. An initial group of rats was used as a control (untreated), and they received only the carrageenan solution. Formulations were applied to the plantar surface (0.125 mg/cm<sup>2</sup>) of the hind paw of the rats by gentle rubbing with the formulation 50 times with the index finger for each treatment, 1 hour before induction of inflammation. The increase in paw volume (oo) was measured using calipers at 0, 1, 2, 3, 4, 5 and 6 h after carrageenan administration. The percent inhibition of oedema induced by carrageenan was calculated for each group using the following equation:

$$\% \text{ swelling} = \frac{V_{\text{control}} - V_{\text{treated}}}{V_{\text{control}}} \times 100$$

## **Data Analysis and Statistics**

Statistical analysis was performed using an ANOVA and Kruskal Wallis non-parametric post-tests using Instat for Macintosh (GraphPad Software Inc, La Jolla, CA, USA)

## **Results**

### **Nanogel synthesis**

Naproxen microgels were successfully synthesized, and mean hydrodynamic diameter for the blank nanogel (BN) particles was found to be  $327 \pm 16.9$  nm and statistically the same as naproxen nanogel (NN) which was  $311 \pm 14.1$  nm. The nanogel adjusted with citric acid to pH 2.5 (NNCA) had larger particle size at  $356 \pm 16.8$ . Treatment of NNCA with sodium carbonate (NNCA+SC) resulted in a statistically significant reduction in particle size to  $289 \pm 12.1$  nm ( $p < 0.05$ ). Representative confocal microscopy and TEM images are shown in Figure 1 which also showed that the nanogels exhibited good monodisperse spheres with regular size. Polydispersity measurements show the lowest value for the blank nanogel (BN), with the highest for NNCA+SC.

## Transdermal delivery

Control BN confirmed there was no naproxen, or co-eluting compound, present in the receptor phases. For the naproxen nanogel prepared under 'standard' conditions (pH 6), NN, the  $J_{ss}$  was  $15.38 \pm 1.67 \mu\text{g cm}^{-2} \text{h}^{-1}$ ; this was 2.5x greater than the control equimolar naproxen solution at pH 6, for which the flux was  $5.3 \pm 1.1 \mu\text{g cm}^{-2} \text{h}^{-1}$ . For NNCA,  $J_{ss}$  was slightly lower at  $12.93 \pm 2.85 \mu\text{g cm}^{-2} \text{h}^{-1}$  although this difference was not statistically significant. However, for NNCA+SC there was a 2.8-fold increase in  $J_{ss}$  at  $42.83 \pm 3.95 \mu\text{g cm}^{-2} \text{h}^{-1}$  which was statistically different,  $p < 0.05$ , compared to both NN and NNCA (Table 1). Acidified solution was not examined because of the low aqueous solubility of the free acid at pH 2.5 (2% ionisation, as  $\text{pK}_a = 4.15$ ). Basified naproxen solution was not examined as this would result in 98.6% ionization of the drug, which is known to result much-reduced flux across skin relative to the free-acid form.

## Tape stripping

Figure 2 shows depth profiles for naproxen using 7.5% cross linker, following the application to porcine ear skin of three test nanogels and naproxen solution. Naproxen was delivered throughout the skin, although significant differences were found between the different nanogels. The nanogel formulation NNCA, followed by SC provided the greatest delivery across all strippings and remaining skin; this was statistically significant in all cases ( $p < 0.05$ ). The nanogel formulation NN (prepared at pH 6) was next highest, and the delivery of naproxen from nanogel NNCA, prepared in the presence of citric acid (pH 2.5), was generally lower. The lowest delivery was from the aqueous solution of naproxen. The rank order was: NNCA+SC > NN =

NNCA+TEA > NNCA > naproxen solution. Summation of naproxen in the strips shows total drug delivered: NNCA+SC 3175 mg, NN 2323 mg, NNCA 1750 mg, naproxen solution 790 mg (Table 1, Figure 3). These figures followed the same trend as for the transdermal delivery.

### **Western Blotting**

Representative Western blots for COX-2 expression in porcine skin dosed with naproxen-loaded nanogels or naproxen solution, are shown in Figure 4. The trend after both 6h and 10h was: Control > naproxen solution > NNCA > NNCA+TEA > NNCA+CA. NNCA (prepared under acidic conditions with citric acid) elicited a greater effect than the equivalent solution, indicating superior delivery. When the applied dose was activated with TEA there was no increase in COX-2 expression. However, when activated by sodium carbonate there was a marked reduction in COX-2 level. The trends are further illustrated in Figure 5.

### **Immunostaining**

Figure 6 shows immunostaining images for the presence of COX-2, where higher presence of COX-2 is visualized as darker staining and lower presence is seen as lighter staining allowing comparative semi-quantitative analysis. After 6 h, the control samples showed the highest levels of COX-2, NNCA was lower, while the activated nanogel NNCA+SC showed the lowest COX-2. The same trend was apparent at 10h, although the NNCA nanogel showed almost as much COX-2 as the control. These trends that support that observed in the Western blotting data.

## **Rat paw oedema**

Carrageenan-induced oedema of the paw causes an up-regulation of COX-2 and an increase in prostanoids<sup>19</sup> resulting in tissue swelling (Figure 7). A plot of % swelling versus time, post carrageenan administration and following application of the nanogels is shown in Figure 7, with maximal swelling after 3h. The blank nanogel control showed generally higher swelling than the naproxen solution control, although not statistically significant. NNCA (naproxen nanogel with citric acid) produced generally lower swelling which was statistically lower than controls at 3h. For the naproxen nanogel with citric acid, then sodium carbonate (NNCA+SC) the swelling lower and statistically significant at 3 and 4h relative to controls. Single-dose administration of naproxen (15 mg/kg) or indomethacin (10 mg/kg) was found to decrease in rat paw oedema volume within 5 h after oedema induction by carrageenan<sup>23</sup> thus the nanogels in the current work appear to have more rapid onset of action.

## **Discussion**

PNIPAM gels are known to de-swell with increased temperature and pH, thus two simultaneous environmentally responsive mechanistic factors were in play. Firstly, PNIPAM has temperature-sensitive phase behavior in water, with a lower critical solution temperature (LCST) at about 32 °C – the average surface temperature of skin. Below this temperature, PNIPAM is hydrophilic and swells in water, while above the temperature, it collapses into a smaller volume and becomes less

hydrophilic as hydrophobic groups are in contact with both water and polymer segments facilitating expulsion of water and drug solubilised therein.<sup>7</sup> However, the data for NNCA nanogels were inferior to those for NNCA activated with sodium carbonate, NNCA+SC. Therefore, temperature responsiveness does not fully account for the observed data, rather, the change in pH appeared to be more important. In terms of the second factor, it is known that PNIPAM gels de-swell with increased pH and Table 1 shows that addition of SC resulted in a collapse of ~19% - figure that agrees well with the 19.9% reported previously.<sup>13</sup>

Preparing the nanogels under acidic conditions ensured two things – firstly that the nanoparticle structures would be formed in their expanded form and secondly, that the entrapped naproxen was in the free-acid form, rather than ionic, which would both inhibit drug leaching into an aqueous environment. When the drug-loaded nanogel was massaged into the skin it would have commenced the penetration process. Subsequent addition of base (sodium carbonate or triethylamine) would have resulted in a pH increase in the local environment of the particles which, assisted by the elevation in temperature from ambient (21 °C) to that of the skin surface (32 °C), instigated nanoparticle de-swelling *in situ*. Thus, a volume equating to ~20% of the absorbed water/drug load would have been released or dumped within the skin in response to such changes in the nanogel structure.

The dose dumping of naproxen could have occurred within the stratum corneum, boosting the drug reservoir hence thermodynamic activity within this tissue which, from Fick's first law, would be expected to enhance the delivery rate into the viable epidermis. Alternatively, dumping may have occurred within the viable epidermis, given rapid penetration from the dose massaged in to the skin surface which, being unionized, would also maximize the penetrability of released naproxen



of the lipophilic domains of the keratinocyte membranes; in the skin, COX-2 is expressed predominantly in suprabasal keratinocytes.<sup>24</sup> However, it is recognized that an unknown amount of naproxen may ionize under such conditions. Either of these processes, or more likely a combination of the two at the same time, resulted in significantly higher levels of naproxen being delivered into the skin. This is evidenced in the fact that tape stripping data, which relates to mass transfer of transfer, reflected the COX-2 and *in vivo* data well, specifically: the greater the mass transfer of naproxen into the viable epidermis, the greater the greater the anti-inflammatory activity by downregulation of COX-2 expression.

Recently porcine skin is a generally accepted as a model for human skin<sup>25,26</sup> and has been used previously as a model to demonstrate anti- and pro-inflammatory effects of a range of topically applied xenobiotics including the steroidal anti-inflammatory drug betamethsone dipropionate and another NSAID, ibuprofen.<sup>18,22</sup> COX-2 is a constitutive enzyme which is upregulated in inflamed tissue where its function is to produce eicosanoids from arachidonic acid in response to the inflammatory stimulus. COX-2 has a short lifespan of 1-2 min at  $V_{\max}$  after which it resolves, becoming permanently inactivated after converting several hundred arachidonic acid molecules to prostaglandins.<sup>27</sup> When the inflammatory stimulus diminishes or in the presence of an anti-inflammatory agent acting at some stage in the cascade<sup>28</sup>, the levels of eicosanoids reduce, causing downregulation in the expression of COX-2. Blockade of COX-2 not only inhibits formation of prostaglandins, leading to reduced pain and inflammation and erythema, it also results in lower COX-2 regulation by feedback.

Thus the data clearly indicate the presence of higher bioavailability of naproxen delivered from the activated nanogel resulting in the downregulation of

COX-2 expression, as observed using both Western blotting and immunostaining. Ideally, confirmatory data on the effect on prostaglandin biosynthesis, particularly pro-inflammatory PGE<sub>2</sub>, would have been useful; however, propionic acid-based ‘profen’ COX inhibitors, including naproxen, are well known to have this effect. Inflammation is a complex physiological response and it would have been interesting to examine the effects of enhanced naproxen skin penetration on lipoxygenase activity and pro-inflammatory TNF- $\alpha$ , IL-1 $\beta$  and IL-6; however, the major pharmacological action of naproxen is as a COX-2 inhibitor so such cytokines would be of lesser relevance.

The widely-used rat paw oedema model provided confirmatory evidence of the percutaneous penetration and COX-2 modulation data in an *in vivo* system – this is in line with other work that has also demonstrated *in vitro* and *in vivo* relationships using this technique.<sup>29</sup> Indirectly, the data also support the biocompatibility of the PNIPAM nanoparticles, and this was explored previously using the COX-2 where no adverse effects were indicated.<sup>30</sup>

The issue of nanoparticle delivery into skin is contentious, with conflicting reports on the ability of such entities to penetrate beyond the stratum corneum.<sup>31</sup> However, we were able to detect PNIPAM nanogels in the receptor phases of Franz diffusion cells<sup>9</sup> having passed through the entire skin membrane; moreover, the topical naproxen delivery and biological COX-2 modulation data presented here can only be explained by the delivery of naproxen to the viable epidermis, with enhanced COX-2 downregulation being a consequence of nanogel particle penetration into the skin.<sup>13,30</sup> We have observed similar results previously using activated nanogels.<sup>14</sup> In this work, the nanogel was massaged into skin where commenced the penetration process.

**Conclusions** Nanotechnology has recognised potential in the management of treating inflammatory disorders.<sup>32</sup> Overall, the current work suggests not only that naproxen-loaded PNIPAM nanogels can target inflamed skin, but also that enhanced biological activity ensues in terms of anti-inflammatory activity, both ex vivo and in vivo following response to environmental stimuli. The system has potential clinical relevance where rapid onset of anti-inflammatory activity is desirable in skin, eg eczema, mild burns, sunburn or other inflammatory conditions such as periodontitis.

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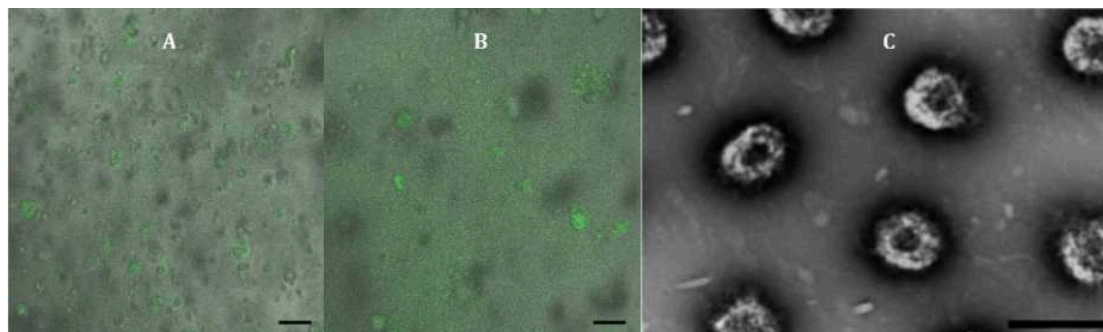
**Table 1** PNIPAM nanogel preparation and percutaneous penetration parameters (n=3)

Abbreviation	Nanogel preparation	Particle size nm	Poly- dispersity	Total naproxen skin content $\mu\text{g cm}^{-2}$	Naproxen $J_{ss}$ $\mu\text{g cm}^{-2} \text{ h}^{-1}$
Nap sol	Naproxen solution (pH 6)	-	-	$990 \pm 167$	$5.3 \pm 1.1$
BN	Blank nanogel (pH 6)	$327 \pm 16.9$	$0.106 \pm 0.04$	0	0
NN	Naproxen nanogel (pH 6)	$311 \pm 14.1$	$0.133 \pm 0.01$	$2323 \pm 646$	$15.38 \pm 1.67$
NNCA	Naproxen nanogel (pH 2.5), no activation	$356 \pm 16.8$	$0.124 \pm 0.04$	$1750 \pm 222$	$12.93 \pm 2.85$
NNCA+SC	Naproxen nanogel (pH 2.5), sodium carbonate activation	$289 \pm 12.1$	$0.172 \pm 0.06$	$3175 \pm 343$	$42.83 \pm 3.95$

**Notes:** Nanogel solution, equimolar with NNCA content; blank nanogel made at pH6; naproxen nanogel is a loaded nanogel following addition of naproxen to reaction mixture. NNCA: naproxen nanogel adjusted to pH 2.5 with citric acid; NNCA+SC: naproxen nanogel adjusted with citric acid to pH 2.5, then activated with 100  $\mu\text{L}$  0.1M sodium carbonate.

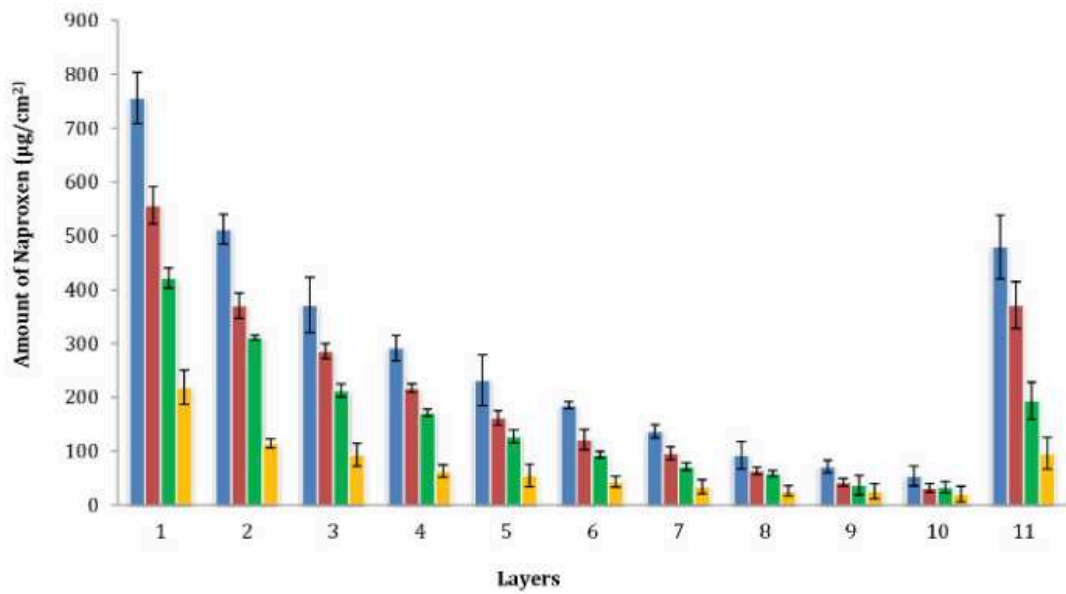


## Figures



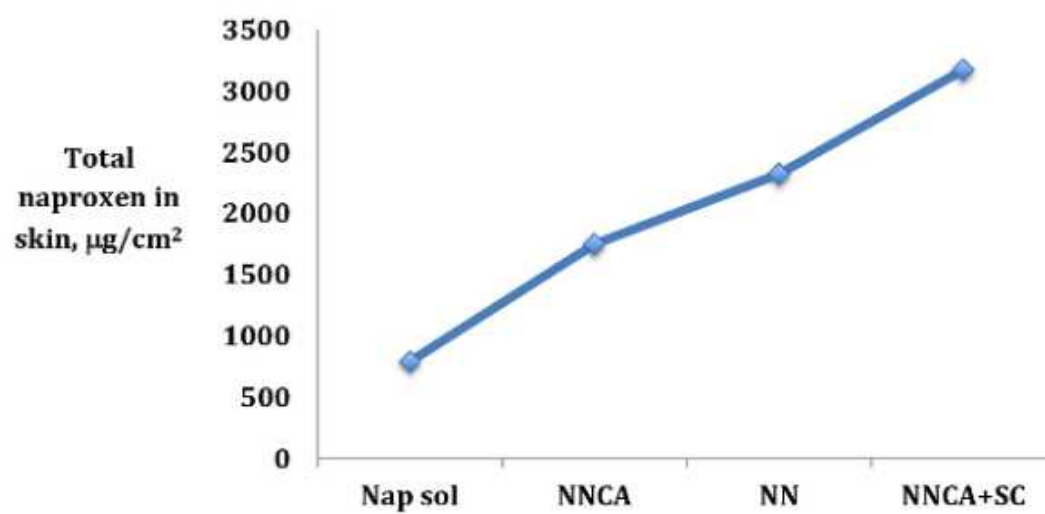
**Figure 1** Images showing PNIPAM nanogels.

**Notes:** A. Confocal microscopy images of naproxen-loaded nanogel prepared at pH 6.0, and B. adjusted with citric acid pH 2.5, B (bar = 500nm) showing size decrease due to pH change. C. TEM image of blank nanogel particles (bar = 500nm).



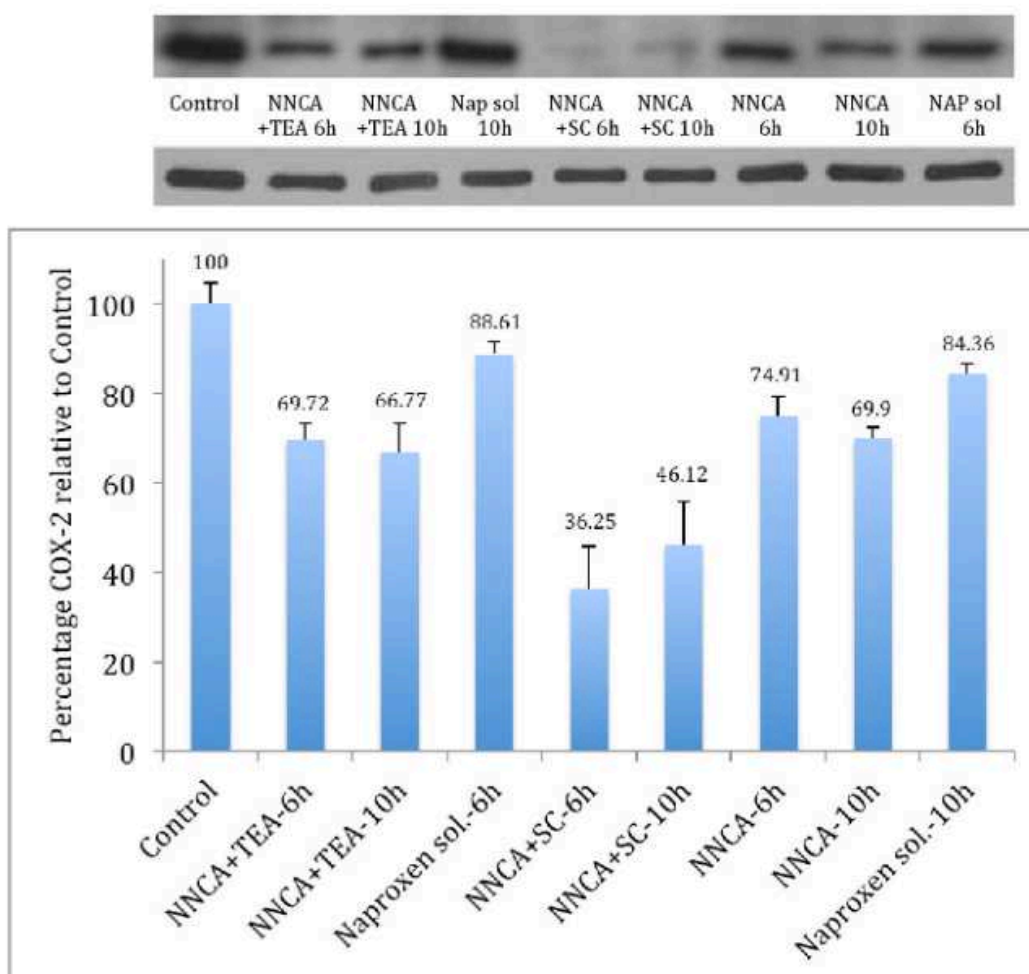
**Figure 2** Depth profiles for the delivery of naproxen into porcine skin from three PNIPAM nanogels and solution of naproxen, 10 strips and remaining skin ( $\mu\text{g}/\text{cm}^2$  versus relative skin depth).

**Notes:** Left to right in each cluster of four: naproxen nanogel with citric acid + sodium carbonate (NNCA+SC) blue, naproxen nanogel (NN) red, naproxen nanogel with citric acid (NNCA) green, naproxen solution, yellow (mean  $\pm$  SD,  $n=3$ ).



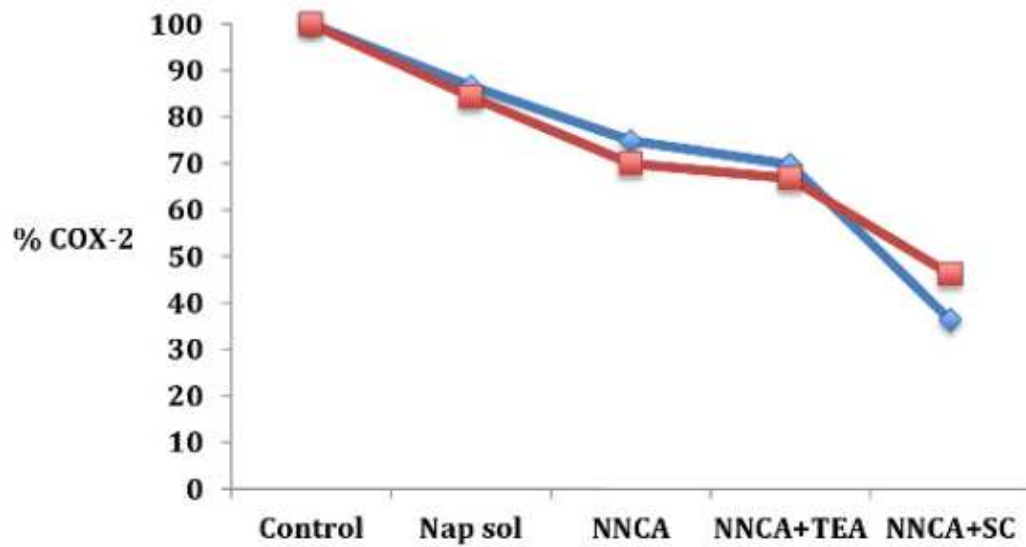
**Figure 3** Total naproxen delivered into skin from the applied formulations ( $\mu\text{g}/\text{cm}^2$ ).

**Notes:** Summation shows total naproxen per square centimetre: NNCA+SC 3175 mg, NN 2323 mg, NNCA 1750 mg, naproxen solution 790 mg.



**Figure 4** Representative Western blots for COX-2 expression following the dosing of porcine skin with naproxen-loaded PNIPAM nanogels after 6 and 10h, relative to water control.

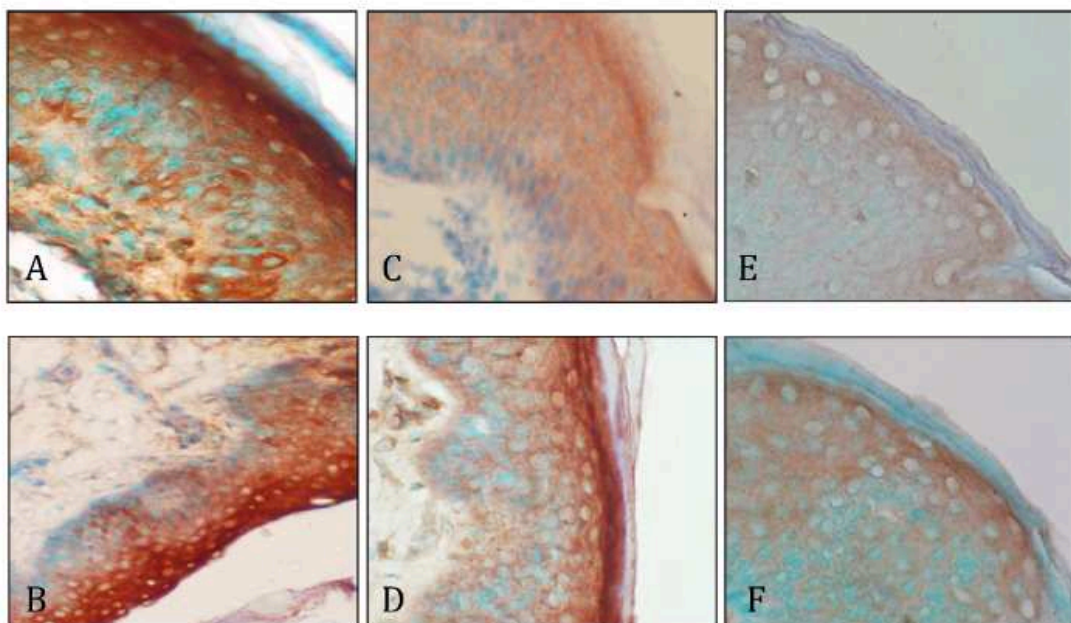
**Notes:** The upper blots show COX-2 for each treatment where the intensity of the bands and is directly proportional to protein level. The lower blots show the  $\beta$ -actin control. The histogram shows relative COX-2 levels as a percentage of the control (mean  $\pm$  SD, n=3) \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .



**Figure 5** Plot showing % COX-2 Western blotting trends at 6h (blue) and 10 (red):

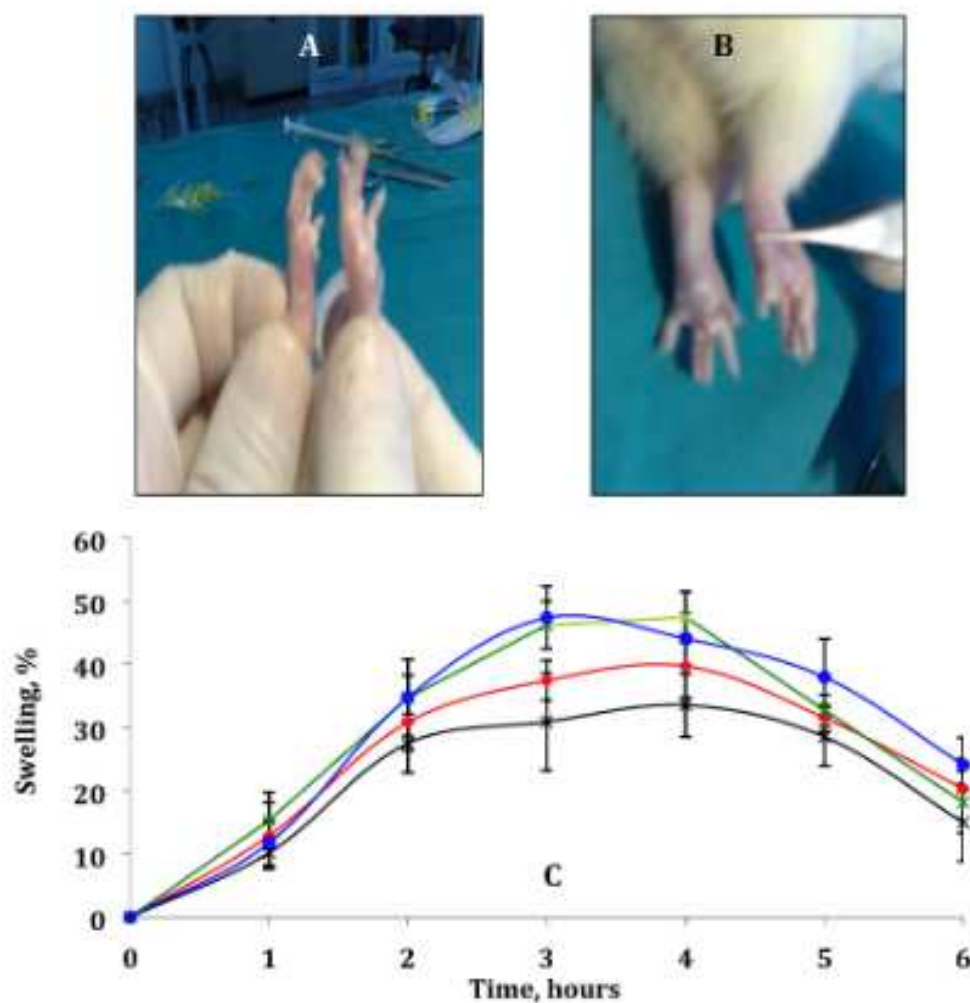
Control > naproxen solution > NNCA  $\approx$  NNCA+TEA > NNCA+SC.

**Notes:** Trend shows: control > naproxen solution > NNCA  $\approx$  NNCA+TEA > NNCA + CA. NB: the lower the COX-2 the greater the anti-inflammatory effect.



**Figure 6** Representative immunostaining images for COX-2 for *ex vivo* porcine skin dosed with PNIPAM nanogels.

**Notes:** *Ex vivo* porcine skin treated with: A. Blank nanogel after 6h, B. Blank nanogel after 10h, C. naproxen nanogel with citric acid after 6h, D. naproxen nanogel with citric acid after 10h, E. naproxen nanogel with citric acid, then sodium carbonate after 6h, F. naproxen nanogel with citric acid then sodium carbonate after 10h.



**Figure 7** Time course of carrageenan-induced oedema in the *in vivo* rat paw test following administration of PNIPAM nanogels.

**Notes:** Results are expressed as % swelling determined at different times after injection of carrageenan in the left hind footpad. A. normal and swollen rat paws, B. application of formulation. C. paw swelling versus time, post carrageenan administration. Green: naproxen solution control, Blue: blank nanogel control, Red: NNCA (naproxen nanogel with citric acid), Black: NNCA+SC (mean  $\pm$  SD, n=3)